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TITLE: LPP is Required for TGF-Beta Induced Motility and Invasion of Neu/ErbB-2 Expressing Breast Cancer Cells

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14. ABSTRACT

Overexpression of the ErbB2 receptor tyrosine kinase is associated with metastatic breast cancer progression and is correlated with poor patient prognosis. Numerous cell-based and transgenic mouse models have demonstrated that ErbB2 and TGFβ signaling cooperatively promote breast cancer cell aggressiveness and metastasis. Nonetheless, the mechanisms underlying the synergy between these two pathways remain unclear. In the present study, we demonstrate that Lipoma Preferred Partner (LPP) is indispensable for mediating TGFβ-induced cell migration and invasion of ErbB2 expressing breast cancer cells. Furthermore, we show that focal adhesion targeting of LPP, through its LIM1 domain, is required for the migratory and invasive phenotypes of ErbB2 positive breast cancer cells in response to TGFß. Using Fluorescence Recovery After Photobleaching (FRAP) techniques, we also determined that LPP is a critical determinant in TGFβ-mediated focal adhesion dynamics and turnover of ErbB2 expressing mammary tumor cells. Together, we have identified LPP as a novel mediator that integrates TGFβ and ErbB2 signaling to promote the migration and invasion of breast cancer cells. Thus, we have further uncovered the mechanisms underlying the synergy between TGFβ and ErbB2 signaling pathways to enhance breast cancer metastasis.

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INTRODUCTION

Overexpression of the ErbB2 receptor tyrosine kinase is observed in 25 to 30% of human primary breast cancer and is associated with poor prognosis. Transgenic mouse models demonstrate that ErbB2 overexpression induces the formation of metastatic breast tumors, and they have also demonstrated a synergy between TGFß and ErbB-2 signaling pathways in promoting breast cancer metastasis to the lung. Although the interaction between the two pathways has been studied in various *in vivo* and cell-based models of breast cancer[1-8], little is known about the mechanisms underlying their cooperation.

We are using NMuMG cells expressing modified forms of an oncogenic ErbB2 receptor: ErbB2(NT) and ErbB2(NYPD). Tumorigenesis and metastasis assays have demonstrated that NT expressing cells aggressively form mammary tumors that are metastatic to the lung. Furthermore, upon TGFß treatment *in vitro*, NT expressing tumor explants display significant increases in motility and invasion that are not observed in the NYPD explants[1].

Using this model system, we identified Lipoma Preferred Partner (LPP) as a novel mediator of TGFß induced motility and invasion of ErbB2 expressing mammary tumor cells. LPP is a nucleo-cytoplasmic protein that mediates processes such as signal transduction, lamellipodia extension, cell adhesion and motility[9-14]. Transient knockdown of LPP in ErbB2(NT) expressing cells abolishes TGFß induced motility and invasion of the mammary tumor cells *in vitro*. LPP has been shown to co-localize with components at the focal adhesion sites and has been implicated in migration of Smooth Muscle Cells. Immunofluorescence experiments revealed that LPP colocalizes to focal adhesion components only following TGFß stimulation of ErbB2(NT) expressing cells, while such colocalization is absent in TGFß stimulated ErbB2(NYPD) expressing cells. Together, our previous data implicates LPP as a novel mediator that is required for TGFß induced motility and invasion of ErbB2 expressing breast cancer cells. *The objective of this award is to further define the mechanisms by which LPP mediates the synergistic interaction between TGFß and ErbB2 signaling pathways to promote breast cancer cell migration, invasion and metastasis.*

BODY

Task #1: Determine whether LPP targeting to focal adhesions is critical in mediating the TGFß-induced migration and invasion of ErbB2 expressing breast cancer cells.

We established an inducible knockdown system using shRNAs targeting the 3' untranslated region (UTR) of LPP. We were able to show that doxycycline-induced knockdown of LPP, using two independent shRNAs, resulted in the complete loss of TGFβ-induced migration (Fig. 1A) and invasion (Fig. 1B) compared to cells that were

not treated with doxycycline or those harboring a control shRNA. Immunoblot analysis confirmed doxycycline inducible knockdowns of LPP with both independent shRNAs and that ErbB2 levels remained similar in all of the cells, regardless of the LPP expression status (Fig. 1C).

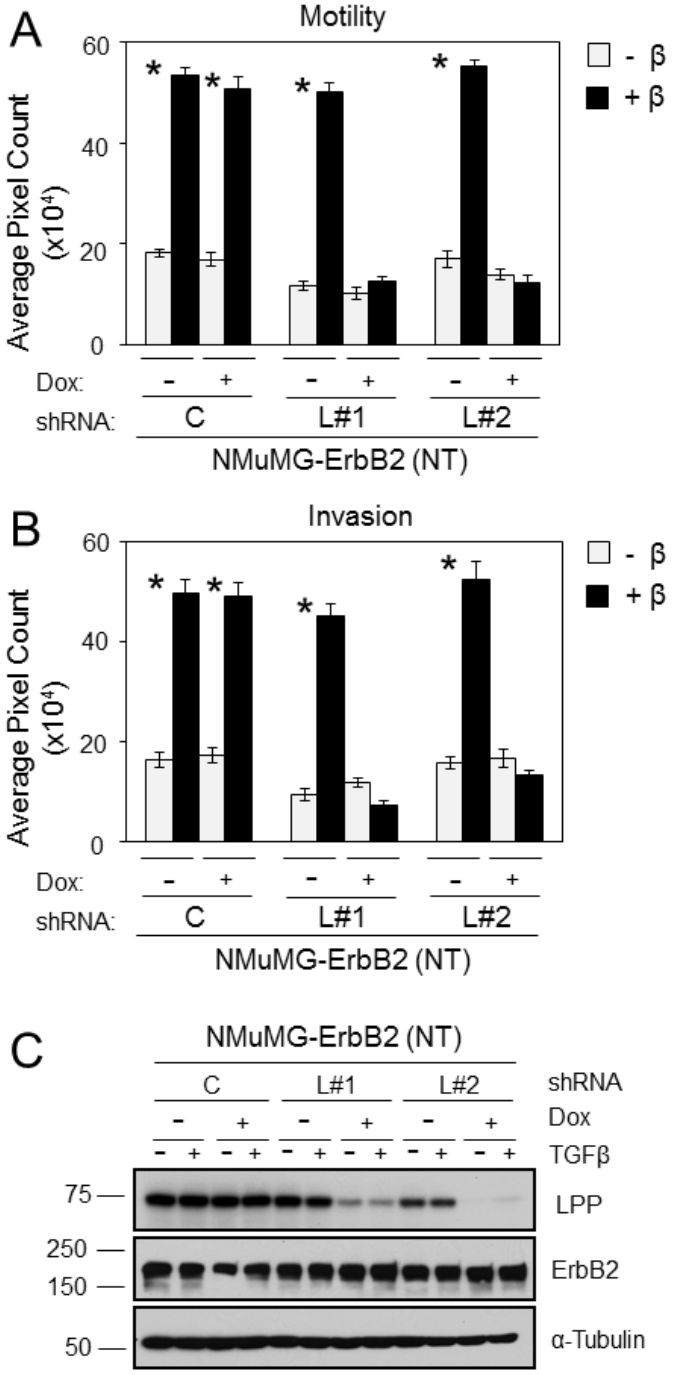

Figure 1. Stable knockdown of LPP abrogates TGFβ-induced migration and invasion of ErbB2-expresing breast cancer cells. NMuMG-ErbB2 cells were engineered to express a luciferase targeting control shRNA (C) or 2 independent shRNAs targeting the 3' UTR of LPP (L#1 and L#2) under the control of a tetracycline inducible system. Cells were incubated with or without doxycycline followed by stimulation with or without TGFβ as indicated. Cells were then subjected to motility (A) or invasion (B) assays using Boyden chambers. The data represents the average of 3 independent experiments performed in duplicate (*; $P < 0.002$). TGFβ-induced cell migration and invasion was completely abrogated in NMuMG-ErbB2 cells that have stably diminished LPP expression by shRNA. (C) Doxycycline treatment effectively diminished LPP expression in cells expressing a LPP targeting shRNA (L#1 and L#2), as assessed by immunoblot. ErbB2 levels remain unchanged regardless of LPP expression and stimulation with doxycycline or TGFβ and α-Tubulin was used as a loading control.

We next determined whether the focal adhesion targeting ability of LPP was required for its ability to promote TGFβ-induced migration and invasion of ErbB2-expressing breast cancer cells. To accomplish this, we generated an EGFP fusion protein with either wild-type LPP (LPP-WT) or a mutant form of LPP that harbors mutations in the first LIM domain (LPP-mLIM1) (Fig. 2A). Immunoblot analysis revealed that endogenous LPP was efficiently reduced in cells following doxycycline treatment and expression of the EGFP-LPP-WT and EGFP-LPP-mLIM1 proteins was readily detected in cells as a slower migrating species, due to the GFP fusion (Fig. 2B). Expression of the EGFP-LPP fusion proteins was confirmed using antibodies against either the GFP or the LPP portion of the fusion protein. Finally, the ErbB2 levels remained uniform across the panel of NMuMG-ErbB2 cells (Fig. 2B). Knockdown of endogenous LPP (VC) resulted in the complete ablation of TGFβ-induced migration (Fig. 2C) and invasion (Fig. 2D). Expression of the EGFP-LPP(WT) fusion protein, but not the EGFP-LPP-mLIM1 mutant, rescued the migration and invasion of NMuMG-ErbB2 cells in response to TGFβ treatment (Fig. 2C, D).

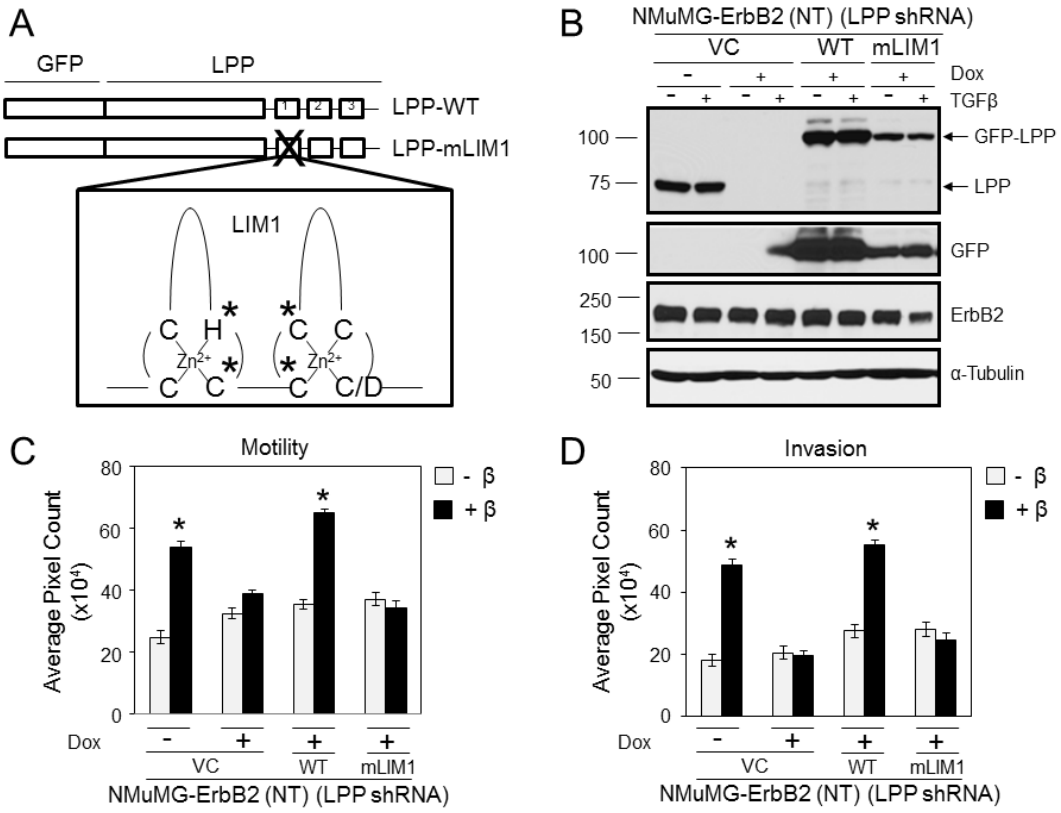

Figure 2. LPP targeting to focal adhesions is critical for TGFβ-induced migration and invasion of ErbB2-expresing breast cancer cells. (A) Schematic diagram of GFP-tagged LPP wild-type (LPP-WT) construct and GFP-tagged LPP construct harbouring mutations to the LIM1 domain (LPP-mLIM1). Amino acid residues marked with (*) were substituted to Alanine. (B) Immunoblot analyses of NMuMG-ErbB2 cells expressing a dox-inducible shRNA against the 3'UTR of

LPP, in which an empty vector (VC), EGFP tagged wild-type LPP (WT) or EGFP tagged LIM1 mutant LPP (mLIM1) are also expressed. Cells were stimulated with or without doxycycline and TGFβ as indicated. Antibodies against LPP and GFP were used to detect the presence of endogenous or exogenous LPP, respectively. ErbB2 levels remain unchanged and α-Tubulin was used as a loading control. NMuMG-ErbB2 cell populations treated with or without doxycycline and TGFβ were subjected to motility (C) and invasion (D) assays using Boyden chambers. Cells were stained and fixed after 24 hours and 5 images were captured from the underside of each transwell. The data is expressed as the average pixel count obtained from 3 independent experiments performed in duplicate (*; $P < 0.001$).

To ensure that the observed effects on migration and invasion that result from LPP loss were not secondary to other TGFβ-induced responses, we also examined proliferation and induction of an EMT in response to TGFβ. We observed no differences in proliferation (Fig. 3A), induction of Smad2 phosphorylation (Fig. 3B) or an EMT (Fig. 3C) in response to TGFβ treatment.

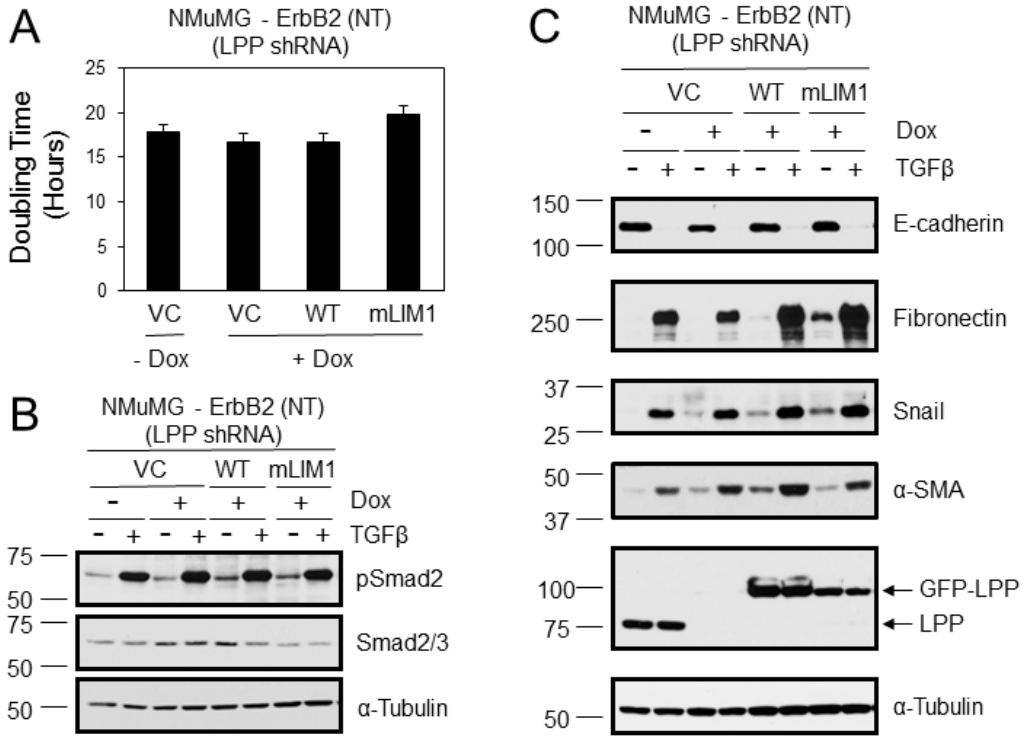

Figure 3. Loss of LPP does not alter NMuMG-ErbB2 proliferation or influence TGFβ-induced EMT. (A) Cell proliferation was assessed using NMuMG-ErbB2(NT) cells, in which endogenous LPP was diminished by LPP-targeting shRNAs, and an empty vector (VC), GFP tagged wild-type LPP (WT) or GFP tagged LIM1 mutant LPP (mLIM1) was introduced. The doubling times are shown and are representative of 2 independent experiments performed in triplicate. (B) The indicated cell populations were stimulated with TGFβ for 30 minutes and

immunoblotting for phospho-Smad2, total Smad2/3 and α-Tubulin was performed. (C) NMuMG-ErbB2 cell populations undergo a TGFβ-induced EMT as shown by the loss of an epithelial marker (E-cadherin) and the gain of mesenchymal markers (Fibronectin, Snail and α-SMA) in response to TGFβ stimulation for 72 hours. The endogenous and exogenously expressed LPP species are indicated and α-Tubulin was used as a loading control.

Moreover, we confirmed that an intact Lim1 domain in LPP is required for localization to Vinculin-positive focal adhesions following TGFβ stimulation (Fig. 4A,B). Together, these results indicate that the ability of LPP to promote enhanced migration and invasion of ErbB2-expressing cells in response to TGFβ requires its proper localization to focal adhesions.

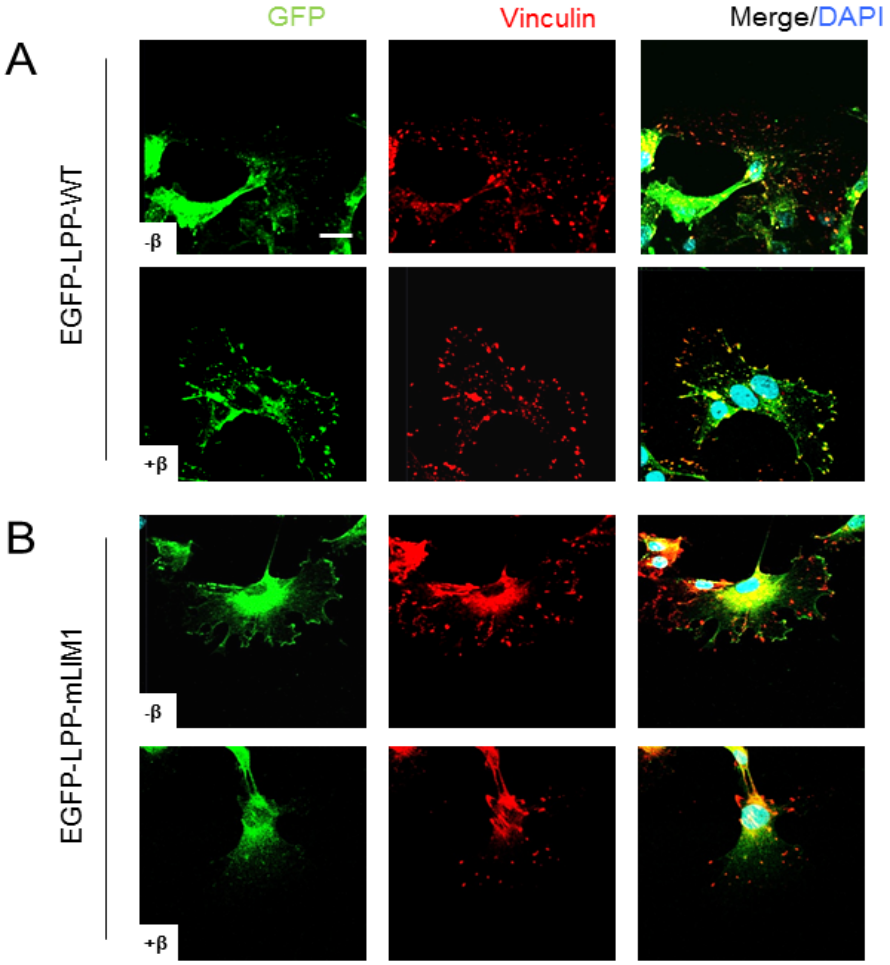

Figure 4. The LPP-Lim mutant fails to target focal adhesions in response to TGFβ. NMuMG-ErbB2 cells lacking endogenous LPP, which express EGFP-LPP-WT (A) or EGPP-LPP-mLIM1 (B) were treated with doxycycline for 72 hours prior to plating onto glass coverslips. Cells were then stimulated with or without TGFβ for 24 hours. A wound was made in the monolayer and cells were cultured for an additional 6 hours. Cells were then fixed and immunofluorescence staining was performed with antibodies directed against GFP to detect exogenously expressed LPP, and Vinculin as a marker of focal adhesions. DAPI was used to mark the nucleus. Representative images are shown (Scale bar = 20 μm for all images).

Task #2: Determine whether LPP mediates focal adhesion turnover of ErbB2 cells in response to TGFß

We previously demonstrated that co-activation of ErbB2(NT) and TGFβ signaling pathways results in the formation of smaller and more numerous focal adhesions, whereas breast cancer cells expressing a signaling defective ErbB2 receptor (NYPD) possessed fewer and larger focal adhesions[1]. We hypothesize that TGFβ and ErbB2 signaling cooperate to enhance focal adhesion turnover and migration. To test this, we employed fluorescence recovery after photo-bleaching (FRAP) to quantitatively assess the kinetics of focal adhesion turnover. High fluorescence recovery after photo-bleaching is indicative of dynamic focal adhesions that are being rapidly turned over. We demonstrate that 57% of the fluorescent signal was recovered after 60s following laser ablation in ErbB2(NT) cells under basal conditions, which increased significantly to 92% following TGFβ stimulation (Fig. 5A). In contrast, 75% fluorescence recovery was observed after 60s in ErbB2(NYPD) cells in the absence of TGFβ, which was reduced to 63% recovery following addition of TGFβ (Fig. 5A).

We further demonstrate that LPP is important for TGFβ-induced focal adhesion turnover. ErbB2(NT) cells retaining endogenous LPP expression (- Dox) exhibited 58% fluorescence recovery after 60s in the absence of TGFβ stimulation, which increased to 99% following addition of TGFβ (Fig. 5B). In contrast, reduced LPP levels (+ Dox) in ErbB2(NT) cells underwent a similar rate of fluorescence recovery (68-72%) irrespective of TGFβ signaling (Fig. 5B). These data support a role for LPP in promoting focal adhesion turnover downstream of the ErbB2 and TGFβ pathways.

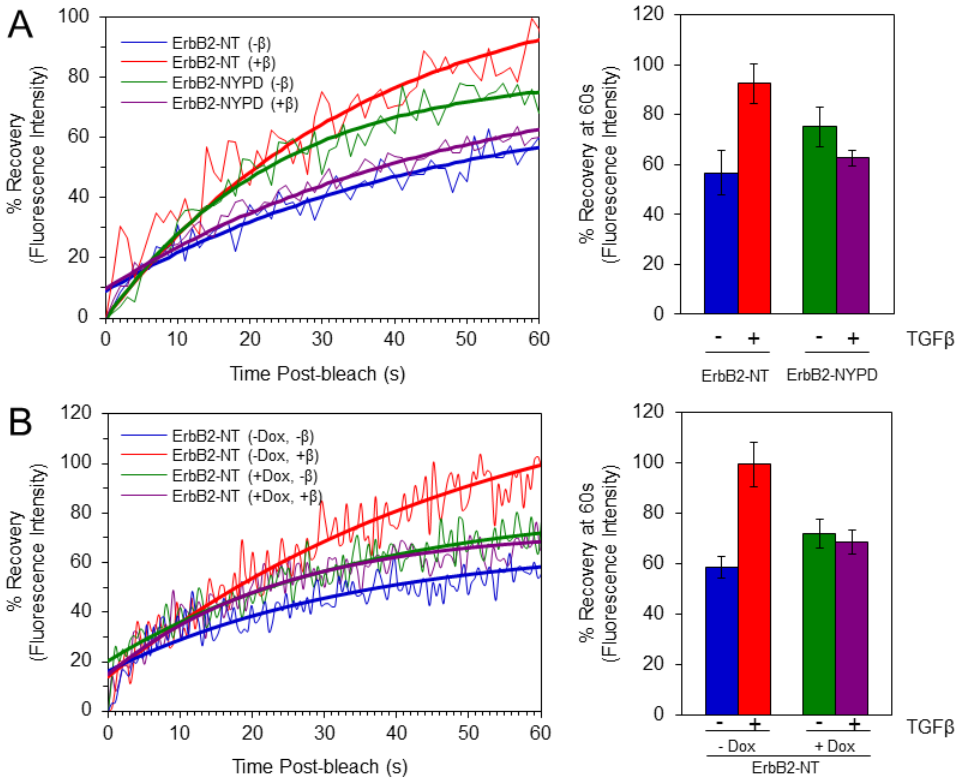

Figure 5. LPP promotes focal adhesion turnover. (A) NMuMG-ErbB2 NT and NYPD cells were transfected with Paxillin-GFP and plated onto glass bottom cultured dishes coated with fibronectin. Cells were stimulated with or without TGFβ for 24 hours prior to Fluorescence Recovery after Photo-bleaching (FRAP) analysis. The raw traces and fitted exponential recovery curves are shown and represent the average of 20-30 adhesions from each cell population. The final % recovery at 60s is also plotted (NT-β: 56.8 ± 9%, NT+β: 92.5 ± 8%, NYPD-β: 76.3 ± 8%, NYPD+β: 62.6 ± 3%). (B) NMuMG-ErbB2-NT cells expressing a LPP targeting shRNA under the control of a dox-inducible system were stimulated with or without doxycycline for 72 hours prior to being transfected with paxillin-GFP. Cells were plated onto glass bottom dishes coated with fibronectin and stimulated with or without TGFβ for 24 hours and then subjected to FRAP analysis. The raw traces and fitted exponential recovery curves are shown and represent the average of 20-30 adhesions from each cell population. The final % recovery at 60s is plotted (-Dox, - β: 58.3 ± 4.3%; -Dox+β: 99.4 ± 8.8%; +Dox, -β: 72.0 ± 5.7%; +Dox, +β: 68.5 ± 4.9%).

KEY RESEARCH ACCOMPLISHMENTS

-We identified shRNA sequences targeting the 3'UTR of *LPP* and established NMuMG-ErbB2 cells expressing a doxycycline inducible system to knockdown LPP.

-We determined that the removal of LPP, by doxycycline induction, abrogates TGFß-induced migration and invasion of ErbB2 expressing breast cancer cells.

-We created eGFP-LPP LIM 1 domain mutant (eGPP-LPP-mLIM1), eGFP-LPP wildtype (eGPP-LPP-WT) and eGFP-vector control (eGFP-VC) constructs

-We determined that eGFP-LPP-mLIM1 cannot target to focal adhesions in response to TGFß compared to its wildtype counterpart (eGFP-LPP-WT). Therefore, the LIM1 domain of LPP is required for its localization to focal adhesions.

-We demonstrated that LPP targeting to focal adhesions is critical for TGFβ-induced migration and invasion of ErbB2-expresing breast cancer cells.

-We established conditions to look at focal adhesion turnover by Fluorescence Recovery After Photobleaching (FRAP).

-We showed that TGFß enhances focal adhesion turnover of NMuMG-ErbB2 expressing breast cancer cells.

-We determined that LPP promotes focal adhesion turnover of NMuMG-ErbB2 breast cancer cells in response to TGFß.

REPORTABLE OUTCOMES

1) Manuscript: **E.Ngan**, J.J. Northey, J. Ursini-Siegel and P.M. Siegel (2012). *An EMT in breast cancer cells engages LPP, a regulator of mesenchymal cell migration and invasion.* Manuscript submitted to the Journal of Cell Science (see appendix for submission confirmation).

2) Abstract and poster presentation: **E. Ngan**, J.J. Northey, and P.M. Siegel. *LPP targeting to focal adhesions in response to TGFβ mediates the migration and invasion of ErbB2-expressing breast cancer cells.* AACR Special Conference: Advances in Breast Cancer Research, 2011 Oct 12-15; San Francisco, California.

3) Abstract and poster presentation: **E. Ngan**, J.J. Northey, and P.M. Siegel. *LPP targeting to focal adhesions in response to TGFβ mediates the migration and invasion of ErbB2-expressing breast cancer cells.* 12th Annual Biomedical Graduate Conference, 2012 February 16; Montreal, Quebec.

4) Seminar presentation: *Defining a role for LPP in the TGFß-induced migratory and invasion phenotype of ErbB2 expressing breast cancer cells*. October 25, 2011. Work-in-Progress Seminar Series. McGill University Goodman Cancer Centre, Montreal, Quebec.

5) Workshop: Attendance and completion of the Montreal Light Microscopy Course was supported by this award.

CONCLUSIONS

Our data to date demonstrate that focal adhesion targeting of LPP is indispensable for TGFβ-induced cell migration and invasion. Together, our results further extend our knowledge of the mechanisms that integrate TGFβ and ErbB2 signaling pathways to enhance breast cancer metastasis. We have completed all of the tasks outlined in the Statement of Work that were described for year 1.

We will continue to investigate the role of LPP in breast cancer. As outlined in the Statement of Work, we will further determine whether LPP is required for breast tumor formation and metastasis to lung using *in vivo* approaches. Furthermore, we will assess whether LPP expression in human breast tumor is associated with clinical outcome. We anticipate that our study will greatly advance our understanding of the mechanisms by which LPP mediates the cooperation of TGFß and ErbB2 signaling pathways to promote breast cancer cell motility, invasion and metastasis.

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APPENDICES

Confirmation of manuscript submission to the Journal of Cell Science

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MS TITLE: An EMT in breast cancer cells engages LPP, a regulator of mesenchymal cell migration and invasion
AUTHORS: Elaine Ngan, Jason J Northey, Josie Ursini-Siegel, and Peter M Siegel

Dear Dr. Siegel

This is an automatic message acknowledging your online submission
to Journal of Cell Science.

Thank you for your submission. We will be in touch as soon as we have some news.

Best wishes,

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